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Transendocardial Delivery of Autologous Bone Marrow Enhances Collateral Perfusion and Regional Function in Pigs With Chronic Experimental Myocardial Ischemia

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OBJECTIVES We tested the hypothesis that intramyocardial injection of autologous bone marrow (ABM) promotes collateral development in ischemic porcine myocardium. We also defined, in vitro, whether bone marrow (BM) cells secrete vascular endothelial growth factor (VEGF) and macrophage chemoattractant protein-1 (MCP-1).

BACKGROUND The natural processes leading to collateral development are extremely complex, requiring multiple growth factors interacting in concert and in sequence. Because optimal angiogenesis may, therefore, require multiple angiogenic factors, we thought that injection of BM, which contains cells that secrete numerous angiogenic factors, might provide optimal therapeutic angiogenesis.

METHODS Bone marrow was cultured four weeks in vitro. Conditioned medium was assayed for VEGF and MCP-1 and was added to cultured pig aortic endothelial cells (PAEC) to assess proliferation. Four weeks after left circumflex ameroid implantation, freshly aspirated ABM ($n = 7$) or heparinized saline ($n = 7$) was injected transendocardially into the ischemic zone (0.2 ml/injection at 12 sites). Echocardiography to assess myocardial thickening and microspheres to assess perfusion were performed at rest and during stress.

RESULTS Vascular endothelial growth factor and MCP-1 concentrations increased in a time-related manner. The conditioned medium enhanced, in a dose-related manner, PAEC proliferation. Collateral flow (ischemic/normal zone × 100) improved in ABM-treated pigs (ABM: 98 ± 14 vs. 83 ± 12 at rest, $p = 0.001$; 89 ± 18 vs. 78 ± 12 during adenosine, $p = 0.025$; controls: 92 ± 10 vs. 89 ± 9 at rest, $p = 0.49$; 78 ± 11 vs. 77 ± 5 during adenosine, $p = 0.75$). Similarly, contractility increased in ABM-treated pigs (ABM: 83 ± 21 vs. 60 ± 32 at rest, $p = 0.04$; 91 ± 44 vs. 36 ± 43 during pacing, $p = 0.056$; controls: 69 ± 48 vs. 64 ± 46 at rest, $p = 0.74$; 65 ± 56 vs. 37 ± 56 during pacing, $p = 0.23$).

CONCLUSIONS Bone marrow cells secrete angiogenic factors that induce endothelial cell proliferation and, when injected transendocardially, augment collateral perfusion and myocardial function in ischemic myocardium. (J Am Coll Cardiol 2001;37:1726–32) © 2001 by the American College of Cardiology

The use of recombinant genes or growth-factors to enhance myocardial collateral blood vessel function represents a new strategy for the treatment of ischemic heart disease (1–9). However, the natural processes leading to collateral development are extremely complex, requiring multiple growth factors interacting in concert and in sequence (10).

We have attempted to develop an approach utilizing natural processes involved in collateral development, even though these are largely unknown. Thus, bone marrow (BM) is a natural source of multiple factors involved in angiogenesis, including fibroblast growth factors 1 and 2 and vascular endothelial growth factor (VEGF) (11,12). In addition, several mediators known to be involved in hematopoiesis may also regulate angiogenesis (13). These multiple cytokines are secreted by several cellular BM components, including stromal cells and various hematopoietic cells (11,13–15). Finally, BM-derived endothelial progenitor cells may be involved in postnatal extramedullary angiogenesis (16,17).

Therefore, in the current investigation we tested the validity of the following hypothesis: "In a porcine coronary occlusion model, direct delivery of freshly aspirated autologous BM (ABM) cells into ischemic myocardium enhances collateral flow delivery and improves myocardial function." We also sought to define whether the BM cells obtained under the specific conditions employed in this investigation secreted potent angiogenic cytokines that may be important cofactors involved in collateral function in ischemic myocardium.

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METHODS

In Vitro Studies

Pig BM culture. Bone marrow cells were harvested from pigs with chronic myocardial ischemia and filtered sequen-

Abbreviations and Acronyms

ABM = autologous bone marrow
BM = bone marrow
H&E = hematoxylin and eosim
LTCM = long-term culture medium
MCP-1 = macrophage chemoattractant protein-1
PAEC = pig aortic endothelial cells
VEGF = vascular endothelial growth factor

tially using 300μ and 200μ stainless steel mesh filters. Bone marrow cells were then isolated by Ficoll-Hypaque gradient centrifugation and cultured in long-term culture medium ([LTCM] Stem Cell Tech, Vancouver, British Columbia, Canada) at 33°C with 5% CO_2. Weekly, half of the medium was removed and replaced with fresh LTCM. The removed medium was filtered (0.2μ filter) and stored at −20°C for subsequent enzyme-linked immunosorbent assay (ELISA) and cell proliferation assays.

Isolation and culture of pig aortic endothelial cells (PAEC). Fresh PAEC were isolated as previously described (18) and cultured using standard techniques. Their identity was confirmed by typical endothelial cell morphology and by positive antifactor VIII staining.

Effects of conditioned medium on PAEC proliferation and DNA synthesis. Pig aortic endothelial cells (passages 3 to 10) were transferred to 96-well culture plates at a density of 5,000 cells/well. Cells were cultured for two to three days before being used in proliferation and DNA synthesis experiments. The conditioned medium of BM cells cultures, collected at four weeks, were pooled from seven culture flasks and used in the assay. Aliquotes (10 μL, 30 μL, 100 μL or 200 μL) of pooled conditioned medium or LTCM (200 μL, as control) were added to confluent PAEC in 96 well plates in triplicate. Four days after culture with conditioned medium or control medium, the PAEC were trypsinized and counted (Coulter Counter Beckman Corporation, Miami, Florida). DNA synthesis was assayed at two days after the same culture conditions. One μCi tritiated thymidine was added to each well. Forty-eight hours later, DNA in PAEC was harvested (Mach III M Tomtec, Hamden, Connecticut); radioactivity was counted by liquid scintillation counter (Multidetector Liquid Scintillation Luminescence Counter EG&G Wallac, Turku, Finland).

Determination of VEGF and macrophage chemoattractant protein-1 (MCP-1) in conditioned medium. The concentration of VEGF in conditioned medium was measured using sandwich ELISA kit (Chemicon International Inc., Temecula, California), and MCP-1 was assayed by sandwich enzyme immunoassay kit (R & D Systems, Minneapolis, Minnesota) according to the manufacturer's directions.

In vivo study. The animal study was approved by the Animal Care and Use Committee of the MedStar Research Institute. The protocol conformed to the tenets of the American Heart Association on research animal use. Fourteen specific-pathogen-free domestic pigs weighing approximately 35 to 40 kg underwent ameroid constrictor implantation around the proximal left circumflex artery. Four weeks later, all pigs underwent: 1) selective left and right coronary angiography, 2) transthoracic echocardiography, 3) regional myocardial blood flow assessment, and 4) electromechanical left ventricular mapping. Following these procedures, all animals received morphine sulfate 3 mg intramuscularly every 8 h to alleviate postprocedural related discomfort.

Treatment groups. In a pilot study, we evaluated the feasibility and safety of transendocardial injection of ABM using a tip-deflecting injection catheter (Biosense-Webster, Diamond Bar, California) in 10 ischemic pigs. Each injection site was marked by adding Fluoresbrite YG 2.0 μm microspheres (Polysciences, Inc. Warrington, Pennsylvania) to ABM in a 1 to 9 ratio. Ten injections of 0.2 ml were evenly distributed approximately 1 cm apart, within the ischemic region and its boundaries (lateral wall, n = 5) and within the nonischemic territory (anterior-septal wall, n = 5). Animals were sacrificed at 1, 3, 7 and 21 days (n = 2 in each time point). Two additional animals were also sacrificed at three weeks after 0.5 ml of ABM injections.

In the second phase, animals were randomized to receive 12 injections of 0.2 ml each of freshly harvested ABM aspirate (n = 7) or a similar volume of heparinized saline (n = 7) directed to the ischemic area and its boundaries in a similar fashion to the pilot study. Heart rate and systemic blood pressure were measured continuously, and left atrial pressure was recorded during the myocardial blood flow studies.

An additional seven animals without myocardial ischemia were studied to determine whether transendocardial injection of ABM into normal myocardium increases regional blood flow. Animals were randomized to injections of ABM (n = 4) or heparinized saline (n = 3) into the lateral wall as described above.

BM aspiration and preparation. Immediately after completion of the baseline assessment, animals underwent BM aspiration from the left femoral shaft using standard techniques. Bone marrow was aspirated from two sites (3 ml per site) using preservative-free heparinized glass syringes (20 U heparin/1 ml fresh BM). The bone marrow was immediately macrofiltered using 300μ and 200μ stainless steel filters, sequentially.

Left ventricular mapping procedure. The procedure has been described in detail (19,20). The reconstructed left ventricular electromechanical maps guided direct transmyocardial injections using previously described electromechanical coupling parameters (21).

Echocardiography study. Transthoracic echocardiography (Hewlett-Packard Sonos 1000) image views at the midpapillary muscle level were recorded at rest and during 2 min of right atrial pacing (temporary electrode, 180/min); fractional shortening measurements were obtained as described

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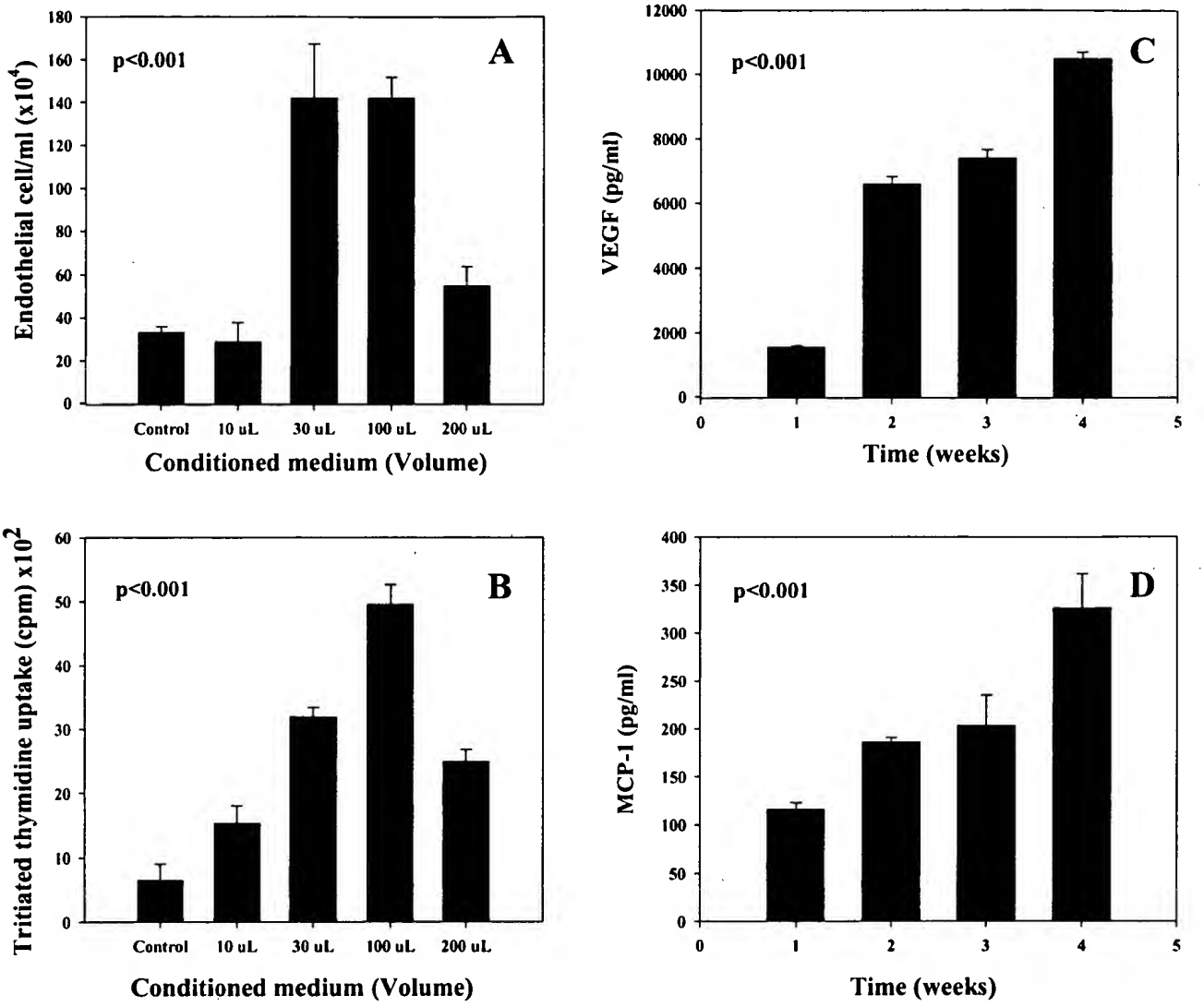

Figure 1. (A) Effects of conditioned medium on PAEC proliferation. Pig aortic endothelial cells were seeded, in triplicate, in 96 well plates for one day; subsequently, the indicated volumes of conditioned medium from pooled samples or control medium were added (with comparable volume of PAEC medium removed). After four days, PAEC, cultured with conditioned medium or control medium, were harvested and counted. (B) Effects of conditioned medium on tritiated thymidine uptake by PAEC. After two days, 1 μCi tritiated thymidine was added to each well, and 48 h later, DNA in PAEC was harvested, and radioactivity was counted. (C and D) Changes in bone marrow cell conditioned medium of VEGF (C) and MCP-1 (D) concentrations at one to four weeks, assayed by ELISA. MCP-1 = macrophage chemoattractant protein-1; PAEC = pig aortic endothelial cells; VEGF = vascular endothelial growth factor.

(21). After being digitized and stored in a frame-grabber board (Kodak ImageVue Compact Version 3.02, Eastman Kodak Company, Pennsylvania), the average of three measurements (taken from three different late-recorded frames) in each examined region was used for analysis.

Angiographic collateral flow assessment. Each coronary angiography study included dedicated injections to detect late collateral dependent filling of the occluded left circumflex artery or its major branches. Angiographic collateral flow was assessed using the Rentrop semiquantitative (0 to 3) score system for both left to left and right to left selective coronary injection (22).

Regional myocardial blood flow. Fluorescent colored microspheres (23) (Interactive Medical Technologies, West Los Angeles, California) were used to assess regional myocardial blood flow at rest and during maximal coronary vasodilation induced by infusing adenosine at a constant rate of 140 μg/kg/min (Fujisawa USA, Deerfield, Illinois). Measurements were quantified by the reference sample technique (24). Two central 7-mm thick slices were each divided into 16 endocardial (n = 8) and epicardial (n = 8) subsegments, as previously described (3,4). The average of eight lateral ischemic zone and eight septal normal zone subsegments measurements was used to assess endocardial and epicardial regional myocardial blood flow. Relative collateral flow was also computed as described (3).

Histopathology. Standard BM smears were prepared before and after propelling freshly filtered ABM aspirate

through the needle using similar injecting pressure as in the in vivo study.

In the pilot study, identified fluorescent-labeled areas were cut into three full thickness adjacent blocks, immersion-fixed and stained with hematoxylin and eosin (H&E) and periodic acid-Schiff. Fluorescent-labeled tissues obtained from the ischemic region were snap-frozen and stained for CD-34, a marker of BM-derived progenitor cells, using monoclonal mouse antibody as primary antibody (Becton Dickinson, San Jose, California).

In the efficacy study phase, full-thickness, 1.5 cm^3 sections from ischemic and nonischemic regions were processed for paraffin sections. Each sample was stained with H&E, Masson's trichrome and factor VIII related antigen. Vascularity, including total area occupied by any blood vessel, the number of these vessels and those with a diameter larger than 50 μm, was assessed, as previously described (25).

Density of endothelial cells was assessed using Sigma-Scan Pro morphometry software (SPSS Science, Chicago, Illinois) and the intensity threshold method. Total endothelial area for each sample and for each specimen was obtained along with relative percent endothelial area (endothelial area/area of myocardium studied). Similar analysis was performed for noninfarcted (viable) myocardium after excluding trichrom-positive stained areas.

Statistics. Unpaired and paired Student *t* tests were used for comparison of nominal and normalized values between and within groups, respectively. Wilcoxon rank-sum test was used when test for normal distribution failed. Repeated measure of variance was used to compare the concentration of VEGF and MCP-1 in the control medium or BM-conditioned medium collected at weeks 1, 2, 3 and 4. One-way analysis of variance (ANOVA) was used to compare the effects of conditioned medium on PAEC proliferation and tritiated thymidine uptake by PAEC. All results are presented as mean ± SD. A p value <0.05 was considered as statistically significant.

RESULTS

In Vitro Study

Conditioned medium-induced proliferation of porcine aortic endothelial cells. The BM-conditioned medium collected at four weeks increased proliferation of PAEC as determined by both direct counting and measuring tritiated thymidine uptake ($p < 0.001$ for both; Fig. 1, A and B) in a dose-related manner. When LTCM was completely replaced by BM-conditioned medium (200 μL), the observed stimulatory effect diminished. Similar dose-related results were observed in the tritiated thymidine uptake studies.

BM cells staining for factor VIII. Limited number (5% ± 4%) of freshly aspirated BM cells stained positive for factor VIII. This contrasted with 57% ± 14% of the adherent layer of BM cells cultured for four weeks, of which 60% ± 23% were endothelial-like cells and 40% ± 28% appeared to be megakaryocytes.

Table 1. Regional Contractility of the Ischemic Wall*

	Baseline	Follow-Up	p Value
Rest			
ABM (%)	60 ± 32	83 ± 21	0.04
Control (%)	64 ± 46	69 ± 48	0.74
Pacing			
ABM (%)	36 ± 43	91 ± 44	0.056
Control (%)	37 ± 56	65 ± 56	0.23

*Fractional shortening measurements were obtained by measuring % wall thickening (end-systolic thickness minus end-diastolic thickness/end-diastolic thickness) × 100, and then the ischemic territory value (lateral area) was divided by the nonischemic value (anterior-septal area).
ABM = autologous bone marrow.

VEGF and MCP-1 in BM-conditioned medium and in control medium. Over four weeks, concentrations of VEGF and MCP-1 in BM-conditioned medium increased gradually to seven and three times the first week level, respectively (Fig. 1, C and D). Vascular endothelial growth factor and MCP-1 levels in a control culture medium, not exposed to BM, were 0 and 11 ± 2 pg/ml, respectively.

In vivo study. Fourteen of 20 pigs completed the study. At intervention, weight was similar between groups (ABM vs. controls = 68 ± 4 vs. 69 ± 4 kg). Five died suddenly within three weeks of ameroid implantation (before ABM injection), presumably from ameroid-induced abrupt coronary occlusion. All animals survived the BM aspiration and catheter-based intramyocardial injections. One animal, randomized to the ABM-treated group, experienced acute myocardial ischemia during ameroid implantation and died seven days before study termination. All animals were hemodynamically stable, and no differences were noted between groups in heart rate, mean blood pressure or mean left atrial pressures at baseline and at four-week follow-up (data not shown).

Myocardial function. Preintervention relative fractional wall thickening, at rest and during pacing, was similar between groups ($p = 0.86$ and 0.96, respectively; Table 1). At four weeks, regional wall thickening improved at rest and during pacing, due to an approximately 50% increase in wall thickening of the ischemic lateral wall. No significant changes were observed in the control animals.

Myocardial perfusion. Preintervention measurements were similar between treated and control groups at rest and during adenosine infusion (Table 2). At four weeks, relative regional transmural myocardial perfusion at rest and during

Table 2. Regional Myocardial Perfusion in Ischemic Animals

	Baseline	Follow-Up	p Value
Rest			
ABM (%)	83 ± 12	98 ± 14	0.001
Control (%)	89 ± 9	92 ± 10	0.49
Adenosine			
ABM (%)	78 ± 12	89 ± 18	0.025
Control (%)	77 ± 5	78 ± 11	0.75

ABM = autologous bone marrow.

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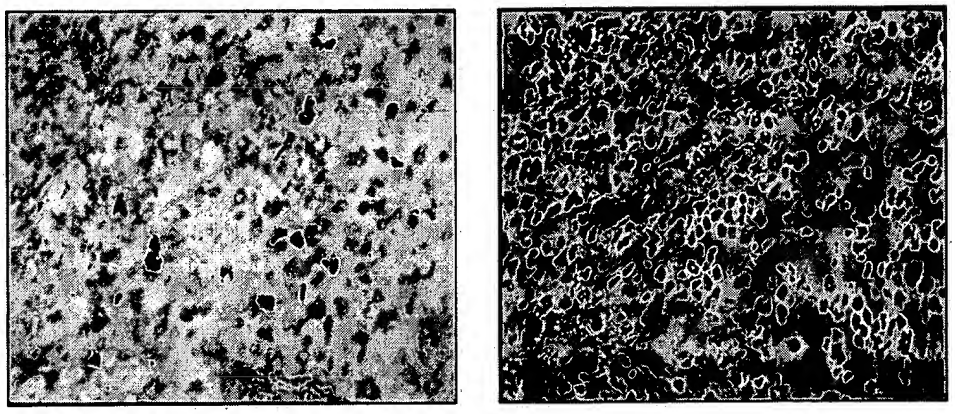

Figure 2. Myocardial injection site stained for CD-34 **(left)** and hematoxylin and eosin **(right)** at three days after transendocardial injection of ABM. Note strong staining for CD-34 in 4% to 6% of the cells.

pacing improved significantly in the ABM-treated group. This was due to an absolute improvement in myocardial perfusion in the ischemic zone both at rest (increase of 57%, $p = 0.08$) and during adenosine infusion (increase of 37%, $p = 0.09$), while no significant changes were noted in absolute flow to the nonischemic zone either at rest (increase of 35%, $p = 0.18$) or during adenosine infusion (increase of 25%, $p = 0.26$). Regional rest myocardial blood flow to the ischemic zone increased both in the endocardial (73%) and epicardial (62%) segments, with somewhat lesser improvement during adenosine infusion (40% in both zones). There were no pre- versus postintervention differences in regional myocardial perfusion in the ischemic and nonischemic zones in the control group.

Injection of ABM into nonischemic myocardium did not improve relative regional myocardial blood flow either under baseline conditions (ABM: 0.92 ± 0.04 vs. 0.95 ± 0.02 at four weeks, $p = 0.27$; control: 0.97 ± 0.04 vs. 0.98 ± 0.04, $p = 0.63$) or during the infusion of adenosine (ABM: 0.93 ± 0.07 vs. 0.95 ± 0.07, $p = 0.54$; control: 1.01 ± 0.09 vs. 1.02 ± 0.16, $p = 0.93$).

Angiographic assessment of collateral flow. Baseline left to left and right to left collateral scoring was similar between groups. At four weeks, no significant improvement was noted in either of the two groups (ABM left to left collaterals: 1.3 ± 1.2 vs. 1.4 ± 1.0 at four weeks, $p = 1.0$ and right to left collaterals 0.7 ± 0.5 vs. 1.0 ± 1.2, $p = 0.46$; control: 2.0 ± 1.2 vs. 2.4 ± 0.8, $p = 0.25$ and right to left collaterals 0.9 ± 1.1 vs. 0.6 ± 0.8, $p = 0.46$).

Histopathology and vascularity assessment. Bone marrow smears before and after passing the filtrated aspirate through the injection catheter revealed normal morphology, absence of macroaggregates and no evidence of cell fragments or distorted cell shapes. Histopathology at day 1 after injections revealed acute lesions characterized by a needle tract containing fibrin and mononuclear cells that morphologically could not be differentiated from BM cells. Cellularity was maximal at three and seven days and declined subsequently over time. At three weeks, more fibrosis was seen in the 0.5-ml injection sites compared with the 0.2-ml sites. Overall, approximately 4% to 6% of the cellular infiltrate showed positive immunoreactivity to CD-34 (Fig. 2).

The ischemic territory in both groups was characterized by small areas of patchy necrosis occupying overall <10% of the ischemic myocardium without evidence for inflammation (Table 3). The nonischemic area revealed normal myocardial structure. There were no differences in total area occupied by any blood vessel or in the number of blood vessels >50 μm in diameter. However, comparison of the total areas staining positive for factor VIII (endothelial cells with or without lumen) revealed a 100% increase in total endothelial cell area in the ischemic collateral-dependent

Table 3. Histopathologic Quantification of Vascularity

	ABM	Control	p Value
Ischemic zone			
No. of blood vessels >50 μm/4 mm²	22.9 ± 15.5	20.1 ± 17.3	0.76
All blood vessels (% area)	4.0 ± 2.5	3.4 ± 2.9	0.69
Endothelial cells (% area)	11.6 ± 5.0	12.3 ± 5.5	0.81
Infarcted tissue (% area)	5.9 ± 7.4	9.1 ± 7.1	0.43
Endothelial cells in noninfarcted tissue (% area)	12.5 ± 5.9	13.7 ± 6.3	0.72
Nonischemic zone			
No. of blood vessels >50 μm/4 mm²	28.2 ± 17.7	16.2 ± 9.2	0.14
All blood vessels (% area)	4.2 ± 3.1	2.8 ± 1.5	0.32
Endothelial cells (% area)	5.7 ± 2.3	8.2 ± 3.1	0.12

ABM = autologous bone marrow.

zone compared with the nonischemic territory (11.6% ± 5.0% vs. 5.7% ± 2.3% area, p = 0.016) of the ABM-treated group, whereas there was no significant difference in the control group (12.3% ± 5.5% vs. 8.2% ± 3.1% area, p = 0.11). Other parameters of vascularity, including percentage of the area occupied by any blood vessel and number of blood vessels >50 μm were similar in the ischemic and nonischemic territories in both groups.

DISCUSSION

In this investigation, we tested the concept that collateral flow may be enhanced by using the natural ability certain cells and tissues have to secrete angiogenic factors in a time-appropriate manner, even though such processes are largely unknown. The results indicate such a strategy is valid.

We found that direct delivery of freshly aspirated ABM into ischemic porcine myocardium enhances collateral flow and improves myocardial function. The concordant changes in these two separately measured, but functionally dependent, parameters of collateral flow adds further support to the conclusion that injection of ABM into ischemic tissue initiates and sustains biologically relevant angiogenic responses. No change in collateral flow was observed when ABM was injected into normal nonischemic myocardium.

In vitro study. In these experiments, we found several possible contributing factors that may mediate BM cell-induced increase in collateral flow. First, BM cells in culture produced large amounts of VEGF, a potent angiogenic growth factor (3,7,10), and MCP-1, which has been identified as a mediator of arteriogenesis, a process whereby remodeling of preexisting larger-sized blood vessels occurs, increasing their capacity to deliver blood (26). In addition, the conditioned medium of BM cells (containing secreted products of the cells) induced proliferation of vascular endothelial cells in a dose-related manner. Whether this effect was caused solely by the increased amounts of VEGF and MCP-1 measured or was also contributed to by other, as yet unknown, molecules is uncertain. In addition, we found in vitro a substantial time-related increase in the number and proportion of BM-derived endothelial cells. It is possible that proliferating endothelial cells derived from the injected ABM contributed to collateral development via incorporation into expanding collateral vessels (16); this concept, however, is still controversial (27). Finally, it is possible that the mild transient inflammatory effect we observed to occur the first few days after the injection of the BM cells may have contributed to the initiation of the angiogenic process.

In vivo studies. The substantial increase in myocardial perfusion we observed was not accompanied by histological evidence of an increased number of capillaries or large blood vessels. Such a discrepancy, also encountered in previous studies (28,29), undoubtedly reflects the very small increases in diameter necessary to effect biologically significant decreases in resistance to collateral flow and the sensitivity of the histologic and angiographic measures we employ to detect such changes, even with detailed methods of angiographic analysis (30). In addition, in the ameroid pig model, standard angiographic assessment may be suboptimal, as extracardiac collaterals, which are not assessed by conventional angiography, account for up to 30% of collateral flow at rest (31).

Catheter-based transendocardial injection had previously been used successfully to deliver solutions such as methylene blue (32) or reporter genes (33), but not cells. The normal BM smears, the positive intramyocardial CD-34 staining at three days after ABM injection and the effects on collateral flow indicate that such an approach does not compromise the function of injected cells. Our study, therefore, establishes, for the first time, the feasibility of a catheter-based transendocardial approach for direct myocardial cell transplantation, a far less invasive procedure than currently available surgical techniques (27).

The evidence that freshly aspirated ABM transplantation into ischemic myocardium is associated with improved collateral flow without adverse effects in the pig model of myocardial ischemia suggests that this strategy may be of clinical importance. Such a strategy would also probably be less costly than many other currently tested angiogenic strategies and would avoid potential toxicity-related issues that, although remote, are definite possibilities with various gene-based approaches using viral vectors.

In summary, the results of this investigation indicate that BM cells secrete potent angiogenic cytokines such as VEGF and MCP-1 and induce proliferation of BM-derived, as well as vascular, endothelial cells. They also show that catheter-based transendocardial injection of ABM is feasible, a strategy resulting in enhanced collateral perfusion and improved mechanical function of ischemic myocardium. These results suggest that clinical studies may now be appropriate to determine whether this novel therapeutic strategy may be an effective way to achieve therapeutic enhancement of collateral development.

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